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In Vivo Regulation of Alternative Pre-mRNA Splicing by the Clk1 Protein Kinase

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Controlled expression of cellular and viral genes through alternative precursor messenger RNA (pre-mRNA) splicing requires serine/arginine-rich (SR) proteins. The Clk1 kinase, which phosphorylates SR proteins, is regulated through alternative splicing of the *Clk1* pre-mRNA, yielding mRNAs encoding catalytically active and truncated inactive polypeptides (Clk1 and Clk1^T, respectively). We present evidence that Clk1 and Clk1^T proteins regulate the splicing of *Clk1* and adenovirus pre-mRNAs in vivo. The peptide domain encoded by the alternatively spliced exon of *Clk1* is essential for the regulatory activity of the Clk1 kinase. This is the first direct demonstration of an in vivo link between alternative splicing and protein kinase activity.

Pre-mRNA splicing occurs in a large macromolecular RNA-protein complex called the spliceosome (19). Reversible protein phosphorylation is essential for both spliceosome assembly and the catalytic process of splicing (9, 17, 18, 24). While there are many phosphoproteins within the spliceosome, it has been widely suggested that the serine/arginine-rich (SR) protein splicing factors (14, 25) are one of the key targets of regulated protein kinase activity (2, 9, 14, 17, 18, 24, 25). SR proteins are essential for both general splicing and the regulation of alternative splicing (1, 7, 16, 29). SR proteins accumulate within subnuclear domains called speckles, where they are believed to be stored or preassembled in spliceosomes (8, 20, 22). The soluble nucleoplasmic pool of phosphorylated SR proteins is found exclusively associated with large nuclear ribonucleoprotein particles. These particles form the active splicing machinery in vivo, and it is believed that variations in the levels of phosphorylation of the SR proteins regulate large nuclear ribonucleoprotein (21, 23, 28). Recent results suggest a role for phosphorylation in the regulation of both SR protein-protein and SR protein-RNA interactions (26).

The Clk1 and SRPK1 kinases are able to phosphorylate SR proteins and cause their redistribution within the nucleus, which implicates both these enzymes in the regulation of SR protein splicing activity (2, 9). Indeed, SRPK1 is able to inhibit β-globin pre-mRNA processing when it is added directly to in vitro splicing extracts (9). We have investigated the role of the Clk1 kinase in the control of pre-mRNA splicing and present evidence that this enzyme regulates the alternative splicing of both its own primary transcript and the adenovirus E1A transcripts in vivo. Our findings suggest that the Clk kinase family may act as an interface between signal transduction pathways and the splicing machinery.

MATERIALS AND METHODS

Plasmid construction. Cytomegalovirus (CMV) *Clk1* and CMV *Clk1^{K190R}* minigenes were generated by replacement of the 236-bp *Nsi*I Clk1 fragments in pECE/M-*Clk1* and pECE/M-*Clk1^{K190R}*, respectively, with the 1,501-bp *Nsi*I fragment from *Clk1-5.6* cDNA. The coding sequence was subsequently cloned into the pcDNA3 expression vector (Invitrogen). Minigene-expressed proteins contain the Myc epitope tag at the amino terminus. CMV *CR-1* was generated by removal of the 709-bp *Cla*I fragment from the Clk1 catalytic domain in the CMV *Clk1* minigene. The resulting expression plasmid generates Clk1^T and a truncated version of Clk1 (amino acids 1 to 179 of Clk1). CMV *CR-2* was generated by digestion of the CMV *CR-1* minigene with *Bam*HI and subsequent blunt ending with DNA polymerase (Klenow fragment), followed by religation with T4 DNA ligase. This introduced a translation stop codon (TGA) following the Myc epitope tag, and therefore CMV *CR-2* does not encode any Clk1 polypeptide. pECE/M-*Clk1$^{\Delta 130–158}$* (where M indicates the Myc epitope tag) was created by PCR-mediated mutagenesis with the following oligonucleotides: 5′CGCACTAG TGTATCGATCAGCTATG3′ and 5′CCCGTGTGAATGGTGCTG3′ to amplify the Myc epitope tag and Clk1 amino acids 1 to 129 and 5′GAGCTCGAGA TATGAAATTGTTGAT3′ and 5′GGCGCACTAGTCGTATGCTTTTTAAGT GG3′ to amplify amino acids 159 to 483. The fragment containing amino acids 159 to 483 (*Xho*I and *Spe*I digested) was cloned into pBK-RSV (*Xho*I and *Xba*I digested) (Stratagene). The fragment containing amino acids 1 to 129 (*Spe*I blunt digested) was then subcloned into pBK-RSV/*159-483* (*Xho*I [T4 DNA polymerase blunted] and *Spe*I digested). The coding sequence was subsequently subcloned into the pECE vector (5). Detailed plasmid construction and maps are available upon request.

Transfections, protein, and RNA isolations. COS-1 cells or 293T cells (3) (60-mm-diameter dish) were transfected as indicated below with Lipofectamine (Gibco BRL) as per the manufacturer's instructions. Filler plasmid DNA was used where applicable to equalize total concentrations of DNA for each transfection. Cells were harvested in phosphate-buffered saline after 24 h and treated as follows. Protein lysates were generated from one-quarter of the cells by the addition of sodium dodecyl sulfate sample buffer. Total RNA was extracted from the remaining three-quarters of the cells by RNeasy purification (Qiagen).

Immunoblots. Immunoblots were probed with 9E10 anti-Myc (6) and E7 anti-β-tubulin (obtained from the American Type Culture Collection) monoclonal antibodies and visualized with horseradish peroxidase-conjugated anti-mouse antibody and by chemiluminescence. Alternatively, anti-hemagglutinin (HA) 12CA5 monoclonal antibody (BABCO Inc.) was used to detect HA-Clk1 polypeptides.

Immunohistochemistry and confocal microscopy. For immunofluorescence, COS-1 cells were transfected, fixed, and stained as previously described (4). Monoclonal antibody 104 (20) was used to detect SR proteins, while 9E10 anti-Myc monoclonal antibodies were used to detect transfected full-length and mutant M-Clk1 proteins. Cells were viewed by confocal microscopy with an upright Leica confocal laser scanning microscope equipped with a 55-mW krypton-argon air-cooled laser and a 63× Plan Apo oil immersion lens.

RT-coupled PCR. For CMV *Clk1*, CMV *Clk1^{K190R}*, CMV *CR-1*, and CMV *CR-2*, 100 ng of RNA was processed for reverse transcription (RT)-PCR by reverse transcribing it and then amplifying it with the following primers: 5′TG GTAGGAGTGGAAGAAG3′ (which corresponds to positions 397 to 414 in the *Clk1* cDNA [11]) and 5′GATGGCTGGCAACTAGAA3′ (which is directed against the bovine growth hormone polyadenylation signal of the pcDNA3 vector). PCR conditions, including the number of cycles and template concentrations, were optimized to maintain linearity during amplification. Linearity was monitored by serial dilution of the template cDNAs, demonstrating a corresponding decrease in signal as well as consistent ratios between bands of interest. PCR products were separated on 1.5% agarose gels, stained with SYBR Green I (Molecular Probes), visualized, and quantitated with a STORM fluorescence imager (Molecular Dynamics). The identities of the spliced *CR-1* mRNAs were confirmed by direct sequencing of the PCR products shown in Fig. 2, in which the larger PCR product is derived from spliced RNA containing an alternatively spliced exon, herein termed exon EB, and the smaller product corresponds to

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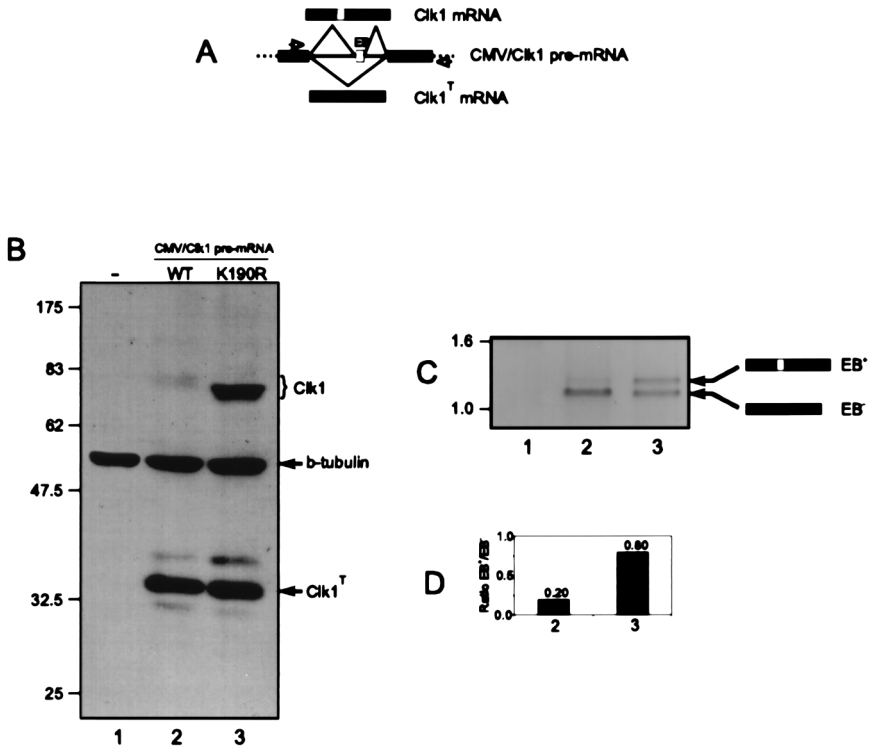

FIG. 1. Catalytic activity of Clk1 kinase regulates splicing of *Clk1* pre-mRNA in vivo. (A) Schematic representation of a portion of the *Clk1* pre-mRNA minigene and the spliced mRNA products. The alternatively spliced exon (exon EB) and primers used for amplification by RT-PCR are shown. CMV, CMV promoter. (B) Clk1 and $Clk1^T$ proteins expressed in COS-1 cells following transfection of the CMV vector (lane 1), CMV *Clk1* pre-mRNA (lane 2), and CMV $Clk1^{K190R}$ pre-mRNA. Clk1 proteins were detected by anti-Myc monoclonal antibody immunoblot analysis. Anti-β-tubulin monoclonal antibody was used to control for equal loadings of proteins. Positions of molecular mass markers (in kilodaltons) are indicated to the left of the panel. WT, wild-type kinase. (C) Detection of *Clk1* mRNAs expressed following RT-PCR amplification. Lanes are as described for panel B. Positions of molecular size standards (in kilobases) are indicated to the left of the panel. (D) Ratios for *Clk1* mRNAs shown in panel C. Lanes are as described for panel B.

spliced RNA in which exon EB has been skipped. RT-PCR analysis to detect E1A-specific mRNAs was as described above with E1A-specific primers (27).

RESULTS

The Clk1 kinase regulates pre-mRNA splicing. We have demonstrated previously that the *Clk1* pre-mRNA is alternatively spliced (4) and that its protein product, the Clk1 kinase, interacts with SR proteins (2). We set out to determine if the Clk1 kinase (11) can elicit changes in the splicing of its own pre-mRNA. To this end we constructed a *Clk1* minigene which contains two introns flanking alternatively spliced exon EB (Fig. 1A). Splicing of the minigene to retain exon EB (exon inclusion) generates an mRNA encoding full-length catalytically active Clk1, whereas splicing to exclude exon EB (exon skipping) generates an mRNA encoding $Clk1^T$ (4). When expressed in COS-1 cells, the Clk1 minigene leads to the production of predominately $Clk1^T$ protein as determined by immunoblot analysis (Fig. 1B, lane 2). In contrast, when a point mutation is introduced within the ATP binding fold of the catalytic domain of the Clk1 minigene, rendering the full-length Clk1 protein catalytically inactive ($Clk1^{K190R}$), significantly more full-length protein is produced (Fig. 1B, lane 3). Analysis of the mRNA produced from the two minigenes, by RT and quantitative PCR amplification, supported the immunoblot results. As can be seen in Fig. 1C and D, lanes 2, cells transfected with the wild-type *Clk1* minigene predominantly expressed mRNA encoding $Clk1^T$ protein. The catalytically inactive $Clk1^{K190R}$ minigene construct produced significantly more mRNA encoding full-length $Clk1^{K190R}$ protein (Fig. 1C and D, lanes 3). These results are consistent with the idea that the catalytic activity of the Clk1 kinase regulates the splicing of the *Clk1* pre-mRNA.

To determine the relative contributions of the Clk1 and $Clk1^T$ proteins to the alternative splicing of *Clk1* pre-mRNA, two smaller minigene constructs were created (CMV *CR-1* and CMV *CR-2* [Fig. 2A]). The *CR-1* minigene contains a large deletion within the catalytic domain and has the capacity to produce only truncated Clk1 proteins and no full-length kinase, while the *CR-2* minigene contains a translational stop codon following the Myc epitope tag which prevents the production of any Clk1-related proteins. The *CR-1* minigene was cotransfected into the human cell line 293T along with increasing amounts of an expression vector encoding a HA epitope-tagged version of Clk1 (HA-Clk1 [2]). The RNA products of the *CR-1* reporter were analyzed by quantitative RT-PCR amplification (Fig. 2). As can be seen in Fig. 2B, lane 2, transfection of the *CR-1* reporter alone gave rise to mRNA products in a ratio similar to that seen with the $Clk1^{K190R}$ minigene (compare Fig. 2B, lane 2, and Fig. 1C, lane 3). Cotransfection of the HA-Clk1 expression vector promoted exon EB skipping. The ratio of exon EB inclusion to exon EB skipping decreased as the HA-Clk1 protein level increased (Fig. 2, lanes 2 to 5). This experiment is representative of five separate transfections which gave 6- to 10-fold decreases in the ratios of exon EB inclusion to exon EB skipping (EB^+/EB^-). Thus, overexpression of Clk1 kinase in vivo can promote exon EB skipping in COS-1 and 293T cells.

Cotransfection of *CR-1* with HA-$Clk1^{K190R}$ resulted in a

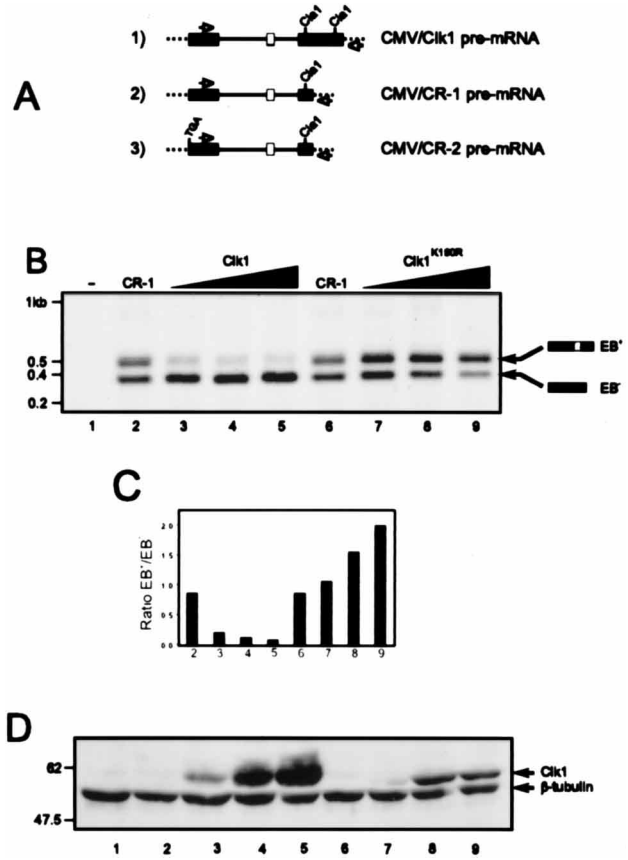

FIG. 2. Catalytically active Clk1 promotes exon skipping of *Clk1* pre-mRNA in vivo. (A) Schematic representation of *Clk1* minigenes. The primers used for amplification by RT-PCR are indicated. (B) Pattern of *Clk1* alternative splicing upon cotransfection of *Clk1* and $Clk1^{K190R}$ expression vectors in 293T cells. Lane 1, CMV vector; lanes 2 to 9, 2.5 μg of CMV *CR-1* plus 0.5, 2.5, and 5 μg of CMV HA-*Clk1* (lanes 3 to 5) or CMV HA-$Clk1^{K190R}$ (lanes 7 to 9), respectively. Positions of molecular size standards are indicated to the left of the panel. (C) Ratios for *Clk1* mRNAs shown in panel B. Lanes are as described for panel B. (D) Detection of Clk1 proteins expressed by anti-HA monoclonal antibody immunoblot analysis. Lanes are as described for panel B. Positions of molecular mass markers (in kilodaltons) are indicated to the left of the panel.

modest (two- to threefold) but reproducible increase in the EB^+/EB^- ratio (Fig. 2B, lanes 6 to 9). One interpretation of this result is that the catalytically inactive $Clk1^{K190R}$ protein can act as a dominant negative inhibitor of the endogenous 293T human Clk1 protein kinase and thus favors exon EB inclusion.

The $Clk1^T$ protein affects splicing. We have previously suggested that $Clk1^T$ may be a natural antagonist to Clk1 activity (4). Since the *CR-1* reporter construct retains the coding capacity to produce $Clk1^T$, we compared the spliced products generated from *CR-1* and *CR-2*, the latter of which produces no Clk1-related proteins. In Fig. 3A it is evident that increasing amounts of *CR-1* transfected per cell favors exon EB inclusion. We believe that this is a result of an increase in the production of $Clk1^T$ protein per transfected cell. To test this idea, a fixed amount of *CR-1* was transfected along with increasing amounts of a $Clk1^T$ expression vector, and again we observed an increase in inclusion of exon EB (Fig. 3C and D). In contrast, increasing amounts of the *CR-2* reporter, which cannot produce Clk protein, does not alter the EB^+/EB^- ratio (Fig. 3A and B). Thus, overexpression of catalytically active Clk1 favors skipping of exon EB while, conversely, $Clk1^T$ promotes inclusion of exon EB.

The exon EB domain affects Clk pre-mRNA splicing and SR protein redistribution. We examined whether regions other than the catalytic domain of Clk1 are involved in the promotion of exon EB skipping. In particular, since portions of exon EB are conserved among the three known Clk family members (4, 10), we created a deletion mutant of Clk1 lacking exon EB (herein referred to as $Clk1^{\Delta 130\text{-}158}$). The $Clk1^{\Delta 130\text{-}158}$ protein,

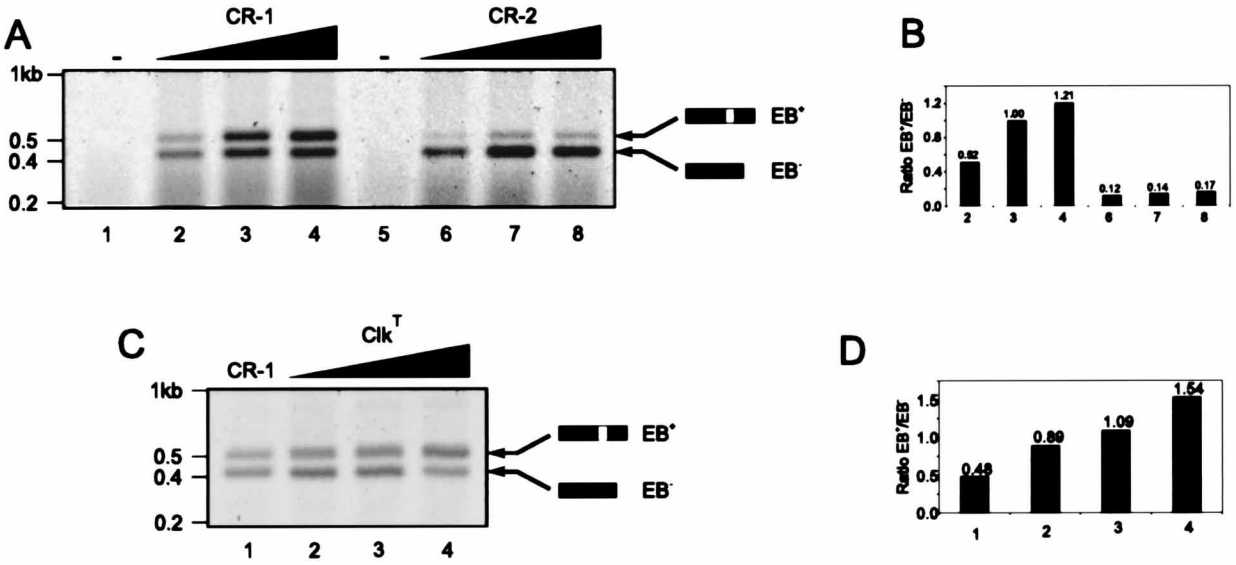

FIG. 3. $Clk1^T$ promotes exon inclusion of *Clk1* pre-mRNA in vivo. (A) Increasing expression of *CR-1* but not *CR-2 Clk1* pre-mRNA promotes exon inclusion. 293T cells were transfected with 0 (lanes 1 and 5), 0.5 (lanes 2 and 6), 2.5 (lanes 3 and 7), or 5 (lanes 4 and 8) μg of CMV *CR-1* (lanes 1 to 4) or CMV *CR-2* (lanes 5 to 8). Positions of molecular size standards are indicated to the left of the panel. (B) Ratios for *Clk1* mRNAs shown in panel A. Lanes are as described for panel A. (C) Pattern of *Clk1* alternative splicing upon cotransfection of the $Clk1^T$ expression vector. Lanes 1 to 4, 2.5 μg of CMV *CR-1* plus 0, 0.5, 2.5, and 5 μg of pECE/M-$Clk1^T$ (4), respectively. Positions of molecular size standards are indicated to the left of the panel. (D) Ratios for *Clk1* mRNAs shown in panel C. Lanes are as described for panel C.

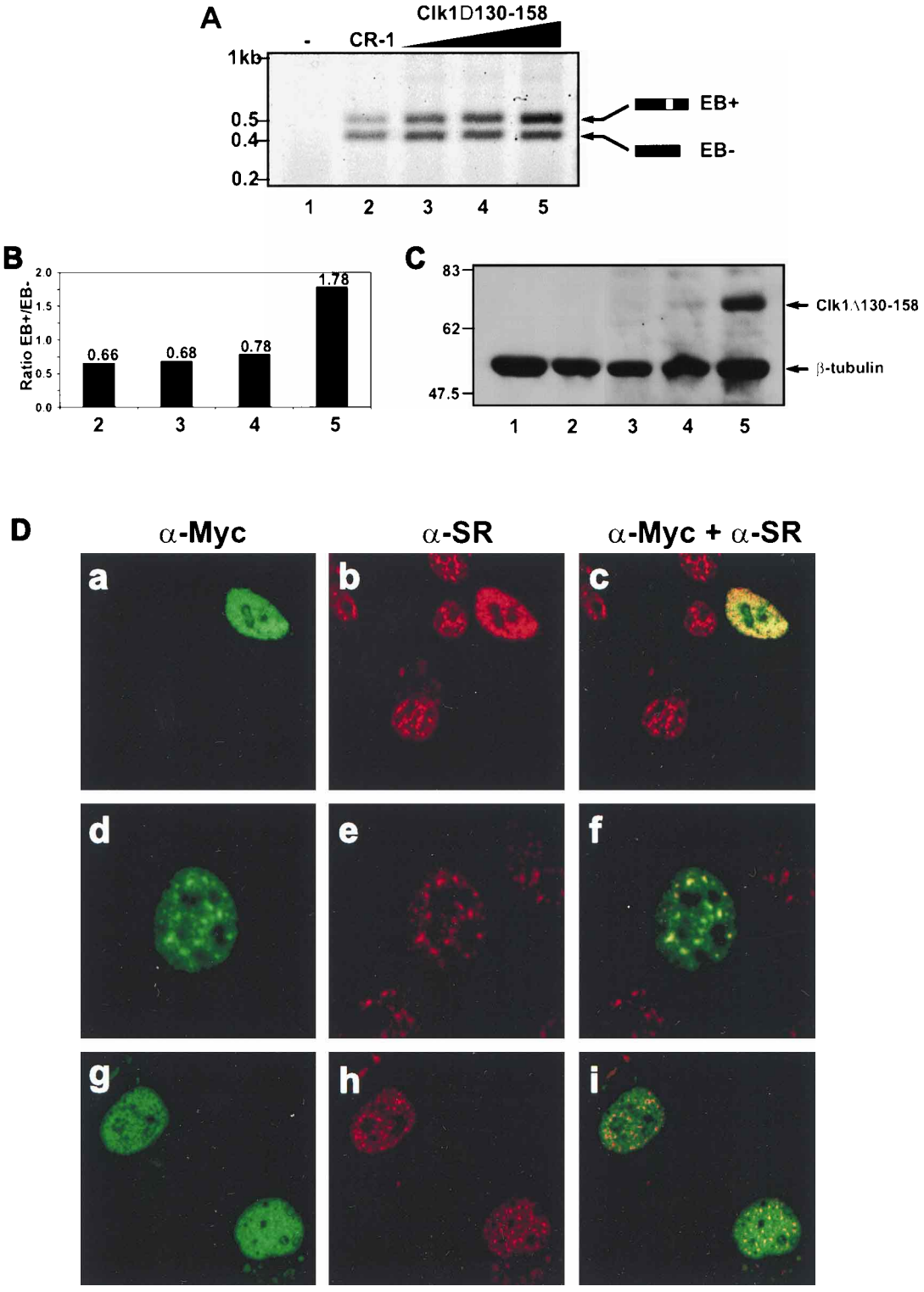

FIG. 4. The peptide motif encoded by the *Clk1* alternatively spliced exon is necessary for exon skipping activity. (A) Pattern of *Clk1* alternative splicing upon cotransfection of the Clk1$^{\Delta 130\text{-}158}$ expression vector. Lane 1, CMV vector; lanes 2 to 5, 0.1 μg of CMV *CR-1* plus 0, 2.5, and 5 μg of pECE/M-*Clk1*$^{\Delta 130\text{-}158}$, respectively. Positions of molecular size standards are indicated to the left of the panel. (B) Ratios for *Clk1* mRNAs shown in panel A. Lanes are as described for panel A. (C) Detection of expression of Clk1$^{\Delta 130\text{-}158}$ protein by anti-Myc monoclonal antibody immunoblot analysis. Lanes are as described for panel A. Positions of molecular mass markers (in kilodaltons) are indicated to the left of the panel. (D) Nuclear localizations of Clk1 isoforms and their effects on the distribution of SR proteins. Indirect immunofluorescent staining of transfected cells with the anti-Myc monoclonal antibody (α-Myc; stains a, d, and g) and anti-SR monoclonal antibody (α-SR; stains b, e, and h) or an overlay of the two signals (α-Myc + α-SR; stains c, f, and i). Cells were transfected with pECE/M-*Clk1* (stains a to c), pECE/M-*Clk1*^{T} (stains d to f), or pECE/M-*Clk1*$^{\Delta 130\text{-}158}$. Magnification, ×56.

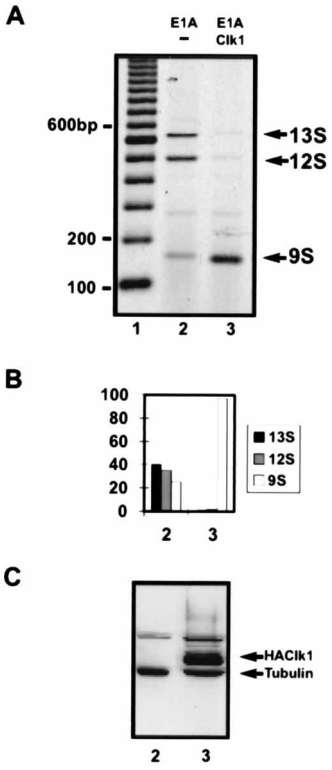

FIG. 5. Effect of Clk1 transient overexpression on alternative splicing of adenovirus E1A pre-mRNA. (A) Pattern of E1A alternative splicing in COS-1 cells upon transfection of the E1A minigene (1, 32) (lane 2) or cotransfection with the pECE/M-*Clk1* expression vector (lane 3). Positions of molecular size standards are indicated to the left of the panel, corresponding the markers in lane 1. (B) The 13S, 12S, and 9S mRNA isoforms were quantitated, and the percentage of each isoform is shown (lanes are as described for panel A). (C) Analysis of protein lysates showed expression of Clk1 in pECE/M-*Clk1*-transfected cells (lanes are as described for panel A).

when expressed in bacteria, displayed considerable catalytic activity, indicating that it retains a functional kinase domain (data not shown). Cotransfection of *CR-1* with $Clk1^{\Delta 130\text{-}158}$ in 293T cells resulted in a splicing pattern similar to that seen with $Clk1^{K190R}$ and $Clk1^{T}$ (Fig. 4A and B).

We have shown here (Fig. 4D, stains a to c) and previously (2) that Clk1 kinase can alter the subnuclear distribution of SR proteins. $Clk1^{T}$ colocalized with SR proteins in nuclear speckles but did not affect their distribution (Fig. 4D, stains d to f). The $Clk1^{\Delta 130\text{-}158}$ protein, which was predominantly nucleoplasmic, like Clk1, but was also found at a low but detectable level in speckles, did not significantly affect SR protein speckles (Fig. 4D, stains g to i). Thus, we conclude that the catalytic activity of Clk1 is necessary but not sufficient for influencing splicing and distribution of SR protein. Furthermore, the peptide motif encoded by exon EB is essential for both SR protein redistribution and exon EB skipping.

Regulation of E1A pre-mRNA splicing by Clk1. To determine whether Clk1 can regulate alternative splicing of other primary transcripts, we cotransfected COS-1 cells with Clk1 and an adenovirus E1A reporter gene which is capable of producing multiple mRNAs (9S, 12S, and 13S) through the use of alternative 5′ splice sites (1, 27). Transfection of the E1A minigene alone generated multiple RNA species characteristic of the utilization of alternative 5′ splice sites, as has been shown previously (1) (Fig. 5, lane 2). The presence of cotransfected Clk1 resulted in a shift in splicing favoring use of the most distal 5′ splice site (9S RNA isoform) (Fig. 5, lane 3), characteristic of the late phase of adenovirus infection (1, 27).

DISCUSSION

Alterations in the concentration of SR protein are thought to be critical to the control of alternative splicing (1, 7, 14, 16, 25, 29). Indeed, exon skipping and inclusion can be modulated by both the concentration of SR proteins and the placement of the splicing enhancers they bind within the pre-mRNA (12, 13). We suggest that the Clk1 kinase phosphorylates an SR protein(s), facilitating its release from storage sites and thus increasing its effective nucleoplasmic concentration and availability to participate in the splicing reaction. This theory is consistent with our previous observations that the subnuclear distribution of SR proteins is regulated in vivo by Clk1 kinase activity. Recently, work from Xu and Manley (26) has demonstrated that in vitro phosphorylation of the SR domain of ASF by the Clk1 kinase directly affects the RNA and/or protein binding properties of SR proteins. Thus, the Clk1 kinase may regulate both the effective concentrations of SR proteins and their activities in the splicing reaction. In support of this idea are our results with the E1A reporter constructs. Previously it has been demonstrated that increases in the concentration of SR protein cause changes in splicing and that, in particular, overexpression of ASF appears to favor use of the proximal 5′ splice site in the E1A gene (1, 15). Since Clk1 overexpression favors the distal 5′ splice site, we conclude that while Clk1 can cause redistribution of SR proteins, it may also modulate the activities of certain SR proteins in the splicing reaction. We suggest that the in vivo effects of Clk1 on SR proteins are via direct phosphorylation; however, we cannot exclude the possibility of indirect effects.

It is interesting that overexpression of Clk1 kinase mimics the switch in splicing of E1A pre-mRNA that is observed during the late phase of infection by adenovirus (1, 27). While the concentrations of SR proteins do not vary during adenoviral replication, SR proteins do appear to be functionally modified at different stages of the infectious cycle (12). We speculate that differential phosphorylation of SR proteins by

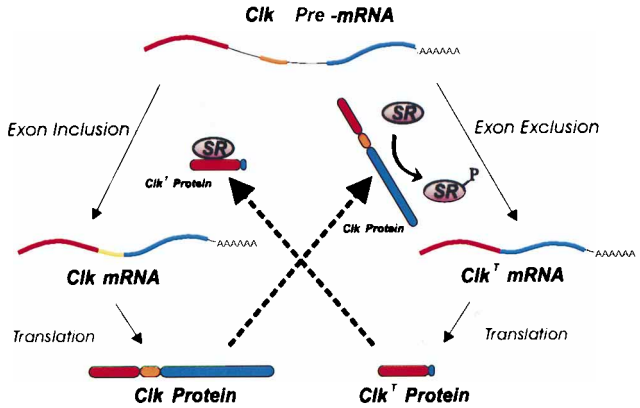

FIG. 6. The Clk kinase regulates splicing of its own pre-mRNA. Splicing of the Clk1 pre-mRNA results in variants either coding for catalytically active (Clk1 kinase) or catalytically inactive ($Clk1^{T}$) isoforms. SR proteins (SR) are known to be involved in alternate splice site selection. The Clk1 kinase phosphorylates an SR protein(s), which then favors exon exclusion, leading to a splice variant coding for the truncated $Clk1^{T}$ protein. $Clk1^{T}$ can associate with but is not able to phosphorylate SR proteins, and therefore, exon inclusion is favored. The resulting splice variant codes for the full-length catalytically active Clk1 kinase. As this form accumulates, the SR protein(s) is phosphorylated, which leads to the eventual decrease in Clk1 in favor of $Clk1^{T}$. P, SR protein phosphorylation.

the Clk kinase may be one mechanism by which viral mRNA splicing patterns are altered during the switch from the early to the late phase of virus replication.

The alternatively spliced exon EB encodes a protein motif which is essential for regulation of alternative splicing by the Clk1 kinase. Thus, while a catalytically active Clk1 kinase domain is necessary for this enzyme to modulate splicing, other Clk domains are also required. It may be that exon EB is essential for substrate recognition by the Clk1 kinase. Indeed, portions of the EB motif are conserved among all three members of the Clk kinase family (4, 10). We have found that a synthetic peptide corresponding to the domain encoded by exon EB is an excellent in vitro substrate of the Clk1 kinase and that some of the Clk1 autophosphorylation sites are found within this exon (4a). Perhaps, as with the interaction of ASF with certain SR proteins, Clk1 interactions are regulated by phosphorylation within this region.

The results presented here suggest a role for the $Clk1^T$ protein in the regulation of pre-mRNA alternative splicing. As shown in the model presented in Fig. 6, increased expression of Clk1 protein influences splicing to generate transcripts encoding $Clk1^T$ protein. Conversely, increased $Clk1^T$ protein favors splicing to generate transcripts encoding catalytically active Clk1 kinase. We have shown previously that $Clk1^T$ and Clk1 can form heterodimers in vitro and suggest that such complexes have either decreased kinase activities or altered substrate recognition properties. It is known, at least at the level of mRNA expression, that Clk1 and $Clk1^T$ isoforms are coordinately expressed and that their relative abundances vary between cell and tissue types (23a and reference 10). While the physiological role of Clk1-$Clk1^T$ heterodimers remains to be established, the results presented here are consistent with a mechanism of regulation of pre-mRNA alternative splicing that is dependent upon Clk1 kinase activity. This key regulatory role for the Clk1 kinase suggests that this enzyme may function as an interface between signal transduction pathways and the mRNA splicing machinery. We speculate that the catalytic activity of Clk1 may be modulated by other phosphatases or kinases which are themselves components of signal transduction pathways, representing a cascade of modifiers responsible for the regulation of splicing.

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P.I.D. and D.F.S. contributed equally to this work.

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